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14. ABSTRACT In this funding period (second of three years), we have completed the first specific as scheduled. We have successfully devised a novel assay system comprise of an engineered 3D vasculature network stably expressing a FRET-based biosensor for myosin light chain kinase (MLCK) activity. This set-up thus offers a very powerful assay system to directly study tumor invasion of the vascular system from the perspective of the endothelial cells. Using this assay, we have observed that endothelial cell MLCK signal is closely mediated by the interaction of the endothelial cells with the invading tumor. Our results implicated a biphasic endothelial signaling response to the interaction with metastatic tumor cells: (1) an initial, general, elevation of MLCK activity above baseline when the cancer cells crawl along the endothelial cell surface, (2) a more acute and marked increase in MLCK activity at the site of tumor entry during active invasion. We also observed that the majority of cancer cells enter the engineered vasculature system via the transcellular route (i.e. through individual endothelial cells), rather than through the paracellular mechanism (through cell-cell junctions), as conventionally believed. We are now actively characterizing the signal of MLCK as well as the reorganization of the actin-myosin network during this event.

15. SUBJECT TERMS
FRET, signal transduction, metastasis, myosin light chain kinase, vasculogenesis

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Introduction

We seek to study in this project the signaling cascades in the underlying endothelium triggered by metastatic breast cancer cells during diapedesis. We proposed to examine these transient and localized signaling events using a three dimensional (3D) assay with superior spatio-temporal resolution. Specifically, we planned to develop and fully integrate 3D fluorescence resonant energy transfer (FRET) studies into 3D tissue engineering to create a vasculature network capable of dynamic read-out of signaling events. This system will allow us to monitor the regional interaction between cancer cells and the endothelial layer. We will use this system to (i) decipher how the metastatic tumor cells increase vascular permeability by controlling the signals that converge on the contractile machinery, (ii) to explore the potential anti-metastatic effects of obliterating these signaling components along the vascular permeability pathway, thus potentially offering new rationale to use the inhibitors as anti-cancer agents.

We have spent the second year of the funding period, as planned, working on the goals outlined in the Statement of Work. We have made remarkable progress simultaneously on several aspects, including (1) refining the method with which we can introduce the breast cancer cells into the 3D matrix with the engineered vasculature network, (2) performing 3D FRET imaging to study the dynamic activity of myosin light chain kinase (MLCK) during active tumor transendothelial migration of breast cancer cells, (3) standardizing protocol to fix and permeabilize the endothelial vasculature in the 3D matrix in order to perform immunofluorescence.

The progress thus far has met the timeline originally planned in the statement of work. However, we have made unexpected discovery of a novel mechanism with which cancer cells invade the vasculature (detailed below). This newly observed phenomenon is vitally important for us to understand how cancer cells penetrate the endothelial barrier, and more importantly may significantly change the paradigm of how we think about tumor invasion. We hereby request

Development planned in Statement of Work

A. Standardizing the conditions for introducing breast cancer cells into the 3D matrix (Months 12-14)

During this funding period, we have tested several ways with which we could introduce metastatic breast cancer cells into the milieu of the engineered vasculature network in the 3D matrix systems. These include the following methods:

(a) Overlaying a gel containing MDA-MB 231 breast cancer cells onto the existing gel with endothelial cells, with the hope that the breast cancer cells would migrate into the bottom gel and invade the vasculature. Unfortunately, while we do observe a small population of breast cancer cell invasion into the gel containing the vasculature, they did not penetrate deep enough into the gel to enter the zone that was covered by the working distance of our objective lens. We also tried directly overlaying the MDA- MB231 cells onto the vasculature-containing gel, and encountered the same problem.

(b) We have injected the fluorescently labeled breast cancer cells into the 3D matrix using either microinjection or Hamilton syringe. We soon realized that microinjection was not the right way to approach this as the tiny needle tips inevitably broke and were clogged as they were inserted into the gel. Hamilton syringe worked well in introducing the breast cancer cells. Even though we initially generated a high local cell density at the site of injection, the breast cancer cells rapidly dispersed into the surrounding area and allowed us to image the transendothelial migration effectively.

(c) Co-culturing MDA-MB231 breast cancer cells with the endothelial cells in the 3D matrix. Despite our initial concern that the presence of breast cancer cells during the assembly of the 3D matrix might interfere with vasculature development, and that it may cause the breast cancer cells to be artificially trapped by the endothelial during vessel development, this method has been the most efficient in introducing breast cancer cells into the matrix. No breast cancer cells can be detected inside the primitive vasculature precursor as the process of lumenization takes approximately 2 days.

Currently, we used the co-culturing and injection methods interchangeably without any detectable difference.

B. Characterization of the fluid exchange rate of the 3D matrix [month 15]

This goal has been accomplished ahead of schedule, and has been reported in the progress report of year 1. Please refer to the report from previous year.

C. Performing 3D FRET imaging to study MLCK activity in endothelial cell during tumor invasion (Months 16-24)

In the indicated time period, we studied the interaction of tumor cells with the per-formed vasculature in details. The main goal of this project is to examine how the breast cancer cells modify various intracellular signals in the endothelium during invasion. In this funding period, we concentrate of the signal pathway mediated by myosin light chain kinase. In

the past year, we have generated the appropriate adenovirus to facilitate the stable expression of a FRET-based MLCK biosensor in the engineered vessel.

C. 1. Dynamic characterization of myosin light chain kinase activity in endothelial cell during tumor invasion

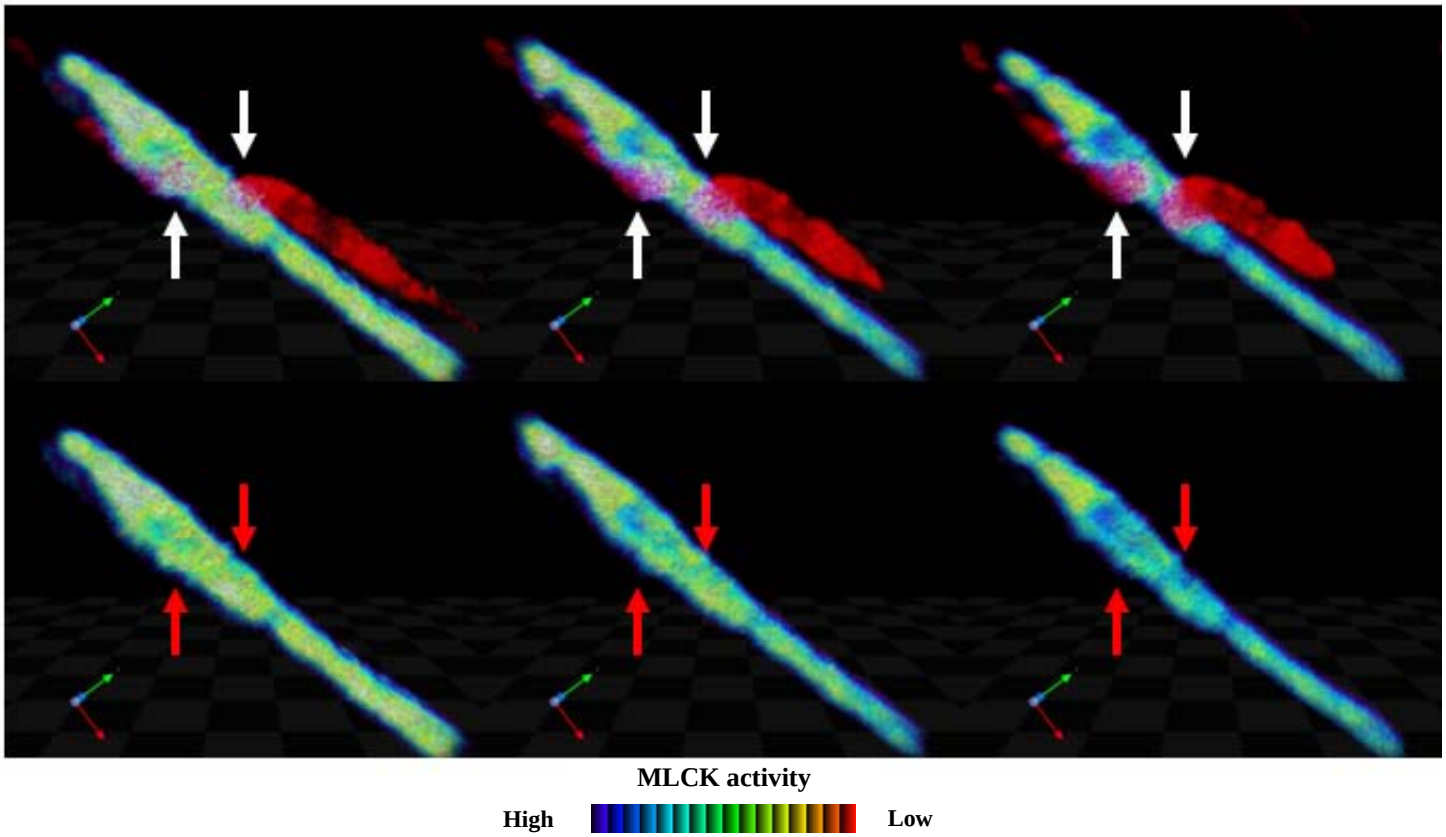

Figure 1 **Metastatic breast cancer cells modulate myosin light chain kinase activity in endothelial cell during invasion into the vasculature.**
(Top Panels): Two individual MDA-MB231 cells (red) simultaneously invading a vessel (ratio colored – from yellow to blue) engineered in 3D collagen matrix. The invasion sites are indicated by white arrows. The vasculature system stably expressed the FRET-based MLCK biosensor. The relative MLCK activity is represented by the ratio below the pictures, with purplish blue representing the active kinase [1]

(Bottom Panels): The identical snapshots of the time-lapsed images as the top panels, but with the red (tumor cell) channel removed from the display, thus highlighting the underlying regional pattern of MLCK activation. Note that MLCK is activated in the areas where the tumor transendothelial migration has occurred. However, the interaction of endothelial cell with the tumor triggers a generalized elevation of MLCK above baseline throughout the cell, with more marked increase in the kinase activity at the invasion site (red arrows).

As shown in figure 1, we have used our novel tumor invasion assay system to dissect the MLCK signals in the endothelium being invaded by metastatic breast cancer cells MDA-MB231. Our preliminary result indicates that the interaction with the cancer cell triggers a general increase in MLCK activity above the baseline, but as the process of invasion continues, the actual site of invasion usually exhibit a marked and localized activation of MLCK. We currently have enough sampling size (N=15) of this event to start preparing for a manuscript.

C. 2. Tumor cells undergo transcellular migration into the vascular system
While most of the adhesion molecules that mediate the interaction and the trapping of tumor cells on the vascular surface have been extensively studied, the actual process by which tumor cells cross the endothelial layer remains poorly understood. Our initial observation showed that tumor cells not only penetrated the endothelial layer by paracellular migration (through cell-cell contact) but interestingly, also through transcellular migration (i.e. through individual endothelial cells). While several reports have documented the phenomenon of transcellular diapedesis by leukocytes through the endothelial layer over the years, the actual transcellular entry of metastatic tumor cell into the vascular system

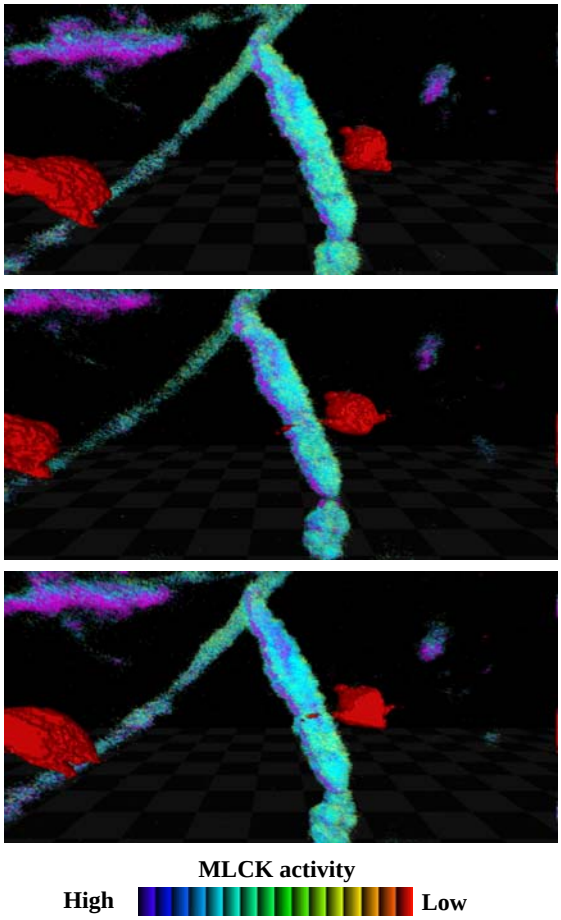

Figure 2 Transcellular invasion of the vascular system by metastatic cancer cells.
Time-lapsed images of an MDA-MB 231 cell (red, in the middle of the images) extending a long pseudopod through the cell body of an endothelial cell (transcellular invasion) rather than through the cell-cell junction (paracellular invasion). Note also the activation of the endothelial MLCK at the site of invasion. Relative activity of MLCK is displayed according to the ratio bar below the image. To ease the visualization of the cancer cell, we displayed the cancer cell using isosurface mode in the Volocity software.

has not been captured live with high spatio-temporal resolution. This tumor-related event was first described by De Bruyn and Cho [2, 3], and unfortunately had since then been largely ignored. Our careful observation of the interaction between tumor and endothelial cells in a 3D milieu clearly highlighted transcellular invasion as a common and alternative mechanism through which tumor cells enter the vascular system. As shown in figure 1, MDA-MB231 breast cancer cells exhibit motile morphology in the 3D matrix rather differently than they do on 2D cell culture dish. While MDA-MB231 typically migrate with fan-like lamella at the leading front when cultured on 2D Petri dishes, they extend long pseudopods while migrating in the 3D collagen matrix – highlighting the importance of studying the process of tumor invasion within environment more closely resemble the physiological condition.

We observed that the tumor cells use these pseudopods to penetrate the endothelial vasculature, consistent with previously described behavior of leukocytes [4-8]. More importantly, as shown in figure 1, the tumor cell sometimes penetrate an endothelial cell rather than through the endothelial junction, a clear evidence of transcellular migration. In light of this newly observed phenomenon, our system has become an extremely powerful platform to directly dissect, with very high spatio-temporal resolution, the transient signaling event(s) within the endothelial cells that may help facilitate transcellular entry of breast cancer cells.

We therefore request at this point to change our second specific aim so that we can characterize this process with greater details. Attached in the appendix are the original statement of work, and the amendment we submitted to the Army Contracting Office Representative. The amended statement of work is pending approval at the time of report submission.

D. Standardizing protocol to fix and permeabilize the endothelial vasculature in the 3D matrix in order to perform immunofluorescence staining. [months 22-24]
This goal has been accomplished ahead of schedule, and has been reported in the progress report of year 1. Please refer to the report from previous year.

Key Research Accomplishments (Cumulative)

- Generation of viral vectors to deliver the FRET sensor constructs to 100% of the endothelial cells used to generate the 3D vasculature
- Standardization of the condition for endothelial cells to reproducibly establish vasculature with lumen in 3D matrix
- Standardization of the condition for endothelial cells expressing the FRET biosensors to establish lumenized 3D vasculature
- Characterization of the fluid exchange rate of the 3D matrix for subsequent drug treatment experiments
- Development of 3D FRET ratio-imaging software module with Improvision, Inc.
- Performing the first 3D FRET confocal microscopy

- Confirmation of the polarization of endothelial cells in the engineered vasculature
- Standardization of cell injection protocol and co-culture condition to introduce the metastatic breast cancer cells into the 3D matrix system.
- Utilizing 3D FRET imaging to dynamically characterize the *in situ* and transient activation of MLCK activity during tumor transendothelial migration.
- Development of an *in vivo* 3D vasculature system expressing FRET sensors which will allow us to examine the signaling events involved in active tumor transcellular entry.

Reportable Outcomes

- 3D FRET and ratio-imaging modules now incorporated in Volocity software (Improvision) version 4.0.1 and onward.
- *Manuscript in preparation*: "Metastatic cancer cells transiently activate myosin light chain kinase *in situ* during transcellular entry into the vascular network: A three-dimensional FRET study in a co-culture system."

Conclusion

We have successfully accomplished all the tasks in the approved statement of work scheduled for the first two years of the 3-year funding period. These accomplishments, taken together, demonstrate that we have completely standardized the 3D assay system with which we can now dynamically monitor, with extremely high spatiotemporal resolution, the transient and *in situ* signaling events within the vasculature network triggered by invading breast cancer cells. We have now completed, on schedule, the first specific aim: *to characterize the signal transduction in endothelial cell in 3D matrix during tumor invasion*.

In the second year of funding, we have accomplished the first specific aim of the proposed project. Using the vasculature network engineered in the 3D matrix, combined with our newly developed time-lapsed 3D FRET imaging techniques, we have studied the process of transendothelial migration of tumor cells with vastly improved spatio-temporal resolution. Our data showed that the interaction with cancer cells caused a general wave of elevation of MLCK activity within the endothelial cells. This is likely due to the calcium release triggered by the engagement of various adhesion molecules on the surface of the endothelial cell [9]. However, the actual process of diapedesis of the tumor cell triggered a marked regional activation of MLCK at the site of invasive entry – implicating a tumor-mediated biphasic Ca^{2+}/calmodulin response in the endothelial cell. We are in the process of further characterizing this biphasic signaling events, as it may be indicative of two related but distinct upstream effectors.

Another critical advancement we have made is the development of the methodology and software module to perform three-dimensional FRET. This microscopy technique will prove to be a powerful improvement not only for those working in breast cancer, but for any investigators who need to expand their FRET imaging repertoire to study dynamic signaling, protein processing, protein-protein interactions as well as protein conformational changes in three dimension either in single cells or in thicker tissue specimens. It also marks the successful combination of advance microscopy technique with *in vitro* tissue engineering.

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APPENDICES

Statement of work

Molecular Mechanisms in Compromised Endothelial Barrier During Breast Cancer Metatasis

We anticipate that the first specific aim will take longer than the second aim because most the technical development and integration have to be first standardized in the first aim. We expect to accomplish the first aim in the first two years.

Task 1 To characterize the signal transduction in endothelial cell in 3D matrix during tumor invasion (months 1-24)

- Obtain FRET-based biosensors for MLCK, Rho, and Rac **[accomplished]**
- Standardizing simultaneous CFP/YFP FRET imaging techniques **[accomplished]**
- Generate adenoviral transfection system for FRET-based MLCK biosensors **[accomplished]**
- Generate adeno- or retroviral vector for FRET-based Rho and Rac biosensors **[accomplished]**
- Design and set up apparatus for casting 3D collagen matrix system in cell culture incubator. **[accomplished]**
- Adapt endothelial cells to 3D matrix for long term growth **[accomplished]**
- Standardizing the condition for endothelial cells to reproducibly establish vasculature with lumen in 3D matrix **[accomplished]**
- Standardizing the condition for endothelial cells expressing the various FRET biosensors to establish vasculature with lumen in 3D matrix **[accomplished]**
- Standardizing the microinjection conditions to introduce MDA-MB 231 and MCF-7 breast cancer cells into the 3D matrix **[accomplished]**
- Characterization of the fluid exchange rate of the 3D matrix, for introducing various inhibitors of ROCK (Rho kinase), MLCK as well as to introduce simvastatin **[accomplished]**
- Performing 3D FRET imaging to study the dynamic activity of MLCK during active tumor transendothelial migration of breast cancer cells. Compare the endothelial response to MDA-MB-231 and MCF-7 **[accomplished]**
- Standardizing protocol to fix and permeabilize the endothelial vasculature in the 3D matrix in order to perform immunofluorescence staining. **[accomplished]**

NOTE: The entire task 1 is now accomplished as planned within the first 24 months of funding.

Original Statement of Work for the 3rd year of Funding Period

Task 2 To delineate the anti-metastatic mechanisms of statins, Rho kinase inhibitor and MLCK inhibitor (months 24-36)

- Standardize the "uncaging" condition of activating CMNB-caged fluorescein in the 3D co-culture matrix. This task will subsequently allow us to measure the permeability of the 3D vasculature. **[months 25-27]**
- Performing fluorescence uncaging experiment to determine if (i) simvastatin, (ii) Y27632 (ROCK inhibitor), (iii) ML-7 (MLCK inhibitor) are capable of preventing VEGF-induced endothelial hyperpermeability **[month 28-29]**
- Determining the inhibitory effects of simvastatin on the dynamic translocation of VEGF-activated Rho and Rac to plasma membrane. **[months 30-31]**
- Performing ratio immunofluorescence experiment to assess the inhibitory effect of (i) simvastatin, (ii) Y27632 (ROCK inhibitor), (iii) ML-7 (MLCK inhibitor) on VEGF-induced myosin phosphorylation. **[months 32]**
- Performing *in situ* cytometric quantification to determine if (i) simvastatin, (ii) Y27632 (ROCK inhibitor), (iii) ML-7 (MLCK inhibitor) can directly attenuate MDA-MB-231 transendothelial migration in the 3D matrix **[months 33-36]**

Amendment to Statement of Work for the 3rd Year of Funding Period (pending approval at the time this progress report is submitted)

Task 2 To delineate the role of myosin II regulatory light chain phosphorylation in tumorigenic transcellular invasion in to the vascular system (months 24-36)

- Confirm transcellular migration event by electron microscopy and live cell imaging with endothelial cells expressing GFP-cadherin. **[months 25-26]**
- Determine the myosin contractile activity in the 3D vascular system at the site of tumor invasion. This can be easily achieved by GFP-tagged myosin regulatory light chain which we have generated years ago. **[months 27-30]**
- Determine the importance of regulatory light chain phosphorylation in mediating the tumor transcellular entrance into the vascular system, using GFP-tagged phosphorylation site mutants of myosin II regulatory light chain. **[months 30-32]**
- Compare the myosin regulatory light chain phosphorylation response to MCF-7 and MDA-MB-231 to probe if the ability to induce myosin activation in endothelial cells can be established as one of the metastatic factors for tumor cells. **[months 33-36]**